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Industry Representative:	Harriet Duncalfe (Berry Gardens)
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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

Culture, DNA extraction and characterisation methods for studying *Fusarium oxysporum* genetics are being optimised. This project aims to reduce strawberry fruit loss by developing tools to accurately identify *Fusarium oxysporum* pathogens.

Background

Fusarium oxysporum is a species complex consisting of both non-pathogenic isolates and pathogenic sub-species known as special forms. The species is categorised into special forms by the range of their hosts. The host range of the pathogen includes over 150 different economically important horticultural and agricultural plant hosts including strawberry, lettuce and potato (Gordon, 2017). *F. oxysporum* is ranked fifth on a list of top 10 fungal pathogens based on scientific and economic importance (Geiser, 2013).

With the rise of monocultures in commercial farming, artificial selection has led to a decrease in genetic diversity in the crop population. This has had a downstream effect on the susceptibility of crops to disease known as “monoculture effect”. This effect has led to an increase the vulnerability of crops to pests and pathogens like *F. oxysporum* (Salaheen, 2019). Symptoms of Fusarium wilt vary slightly between host crops, a recurring symptom is the wilting of plant stems. *F. oxysporum* f sp. *fragariae,* the strawberry pathogen, causes root rot, vascular wilt and crown rot in strawberry fruit. Fusarium wilt was first described in Australia in 1962 (Winks, 1965) and has since been prevalent in Europe, Asia and the Americas. In the USA, California has reported cases of Fusarium wilt in all major production sites which contributes to over 80% of fresh strawberry produced in the country. *F. oxysporum fragariae* is a threat to the $2.22 billion dollar strawberry industry (Agriculture, 2019), in which resistant cultivars are grown however, most commonly grown cultivars are susceptible.

Currently, *F. oxysporum fragariae* is not present in the UK, but it is still a potential threat. F. oxysporum *lactucae* race 4 (lettuce pathogen) was reported during glasshouse production of lettuce in the UK in 2016. A grower in Ireland reported 10% of crops were affected but losses were more severe in subsequent crops reporting up to 100% losses (Taylor A. J., 2019). The value of lettuce grown in the UK is £167 million per year (DEFRA, 2019). If not properly managed, *F. oxysporum lactucae* could have severe impacts on crop yield and thus profits for growers.

Currently, there is no cultural mechanism completely effective against combating *F. oxysporum.* As a soil-borne pathogen, soil fumigation is used to remove it from the infected

soils after infected crops are uprooted. Asexual spores produced by the fungus can survive in soil in the absence of a host for up to six years, which makes its control very difficult (Upasani, 2016). The durability of these spores allows some to remain in the soil unaffected by fumigation meaning they can still pose an issue for crops that will be replanted in the soil.

This research project aims to develop a quick and cost-effective model pipeline using *F. oxysporum fragariae* and *lactucae* to make identification of pathogens in the laboratory and in the field more accurate and less laborious. The early and accurate diagnosis of the pathovar will allow time-sensitive countermeasures to reduce the risk of the pathogen spreading. Fusarium wilt can be a recurring issue causing significant financial losses for growers in trying to manage it in soil, which can become a problem during the propagation chain. This project aims to design a model for accurate identification of Fusarium wilt pathogens which could later be applied to other crop pathogens. Early identification will allow effective management strategies to be implemented earlier to increase crop yield.

Summary

In the first year of this project, methods for extracting DNA from different pathovars have been compared and are being refined. The optimum sampling timing was investigated using growth curve experiments. The genome of the lettuce pathogen, *F. oxysporum lactucae* (race 1), has been sequenced and is being concatenated with previous sequence data. At present, it is being analysed using computer programs. Further work will include conducting pathogenicity tests on *F. oxysporum fragariae* isolates of which have unknown pathogenicity. This test will provide information on the differences in the susceptibility of each cultivar depending on the pathovar.

Financial Benefits

In this stage of the project, there is no financial benefit.

Action Points

There are no action points for growers as the project is still at an early stage.

SCIENCE SECTION

Introduction

Fusarium wilt is a fungal disease caused by *Fusarium oxysporum f sp.* invading host plant roots through the soil. This leads to root rot, vascular wilt and eventually crown rot. These formae speciales are highly host-specific to susceptible crops causing irreparable damage and ultimately, plant death (Taylor A. A., 2019). This makes the pathogen economically important as it affects yield and trade in the agricultural industry.

The genome of *F. oxysporum* pathogens is comprised of two parts: the conserved region and the Lineage-specific (LS) chromosomes. LS regions in the genome have distinct evolutionary profiles which are found in one specific taxonomic group but have negligible or no similarity with genes from other lineages (Zhang, 2015). Pathogen specific (PS) regions are contained within LS chromosomes. The PS region contains genes which allow the fungi to produce toxins and proteins which are used to infect the host plant. These proteins are called effectors. Due to the organisation of the LS region, mutations in the DNA occur at a faster rate than the conserved region. These mutations also cause changes in effector proteins increasing the chances of the pathogen adapting to a different host cultivar (Henry, 2020). Some effectors are recognised by the immune system of the host plant if it carries the necessary resistance gene. Fungal pathogens may gain or lose effectors through mutations to subvert immune response from plants avoiding the effector driven immunity response.

The susceptibility of the host cultivar is dependent on both the complement of resistance genes and the effectors present in different isolates within *F. oxysporum* f. sp. The presence of these effectors defines the aforementioned race structure as it coincides with what pathovar can infect each cultivar.

The dynamic mutations and high rate of virulence have made *F. oxysporum* a global food security threat (Puri, 2019). Working proactively to develop an understanding of the pathogen gives time to prepare for a potential outbreak, develop fast methods of pathogen identification and effective preventative measures. With this in mind, growers will require an accurate and quick diagnostic method for verifying the identity of a pathogen. This test will have to be cost-effective for growers and be able to perform in a glasshouse or on the fields to excise the requirement of a lab.

Major gene resistance and pre-plant fumigation can provide effective control in soil-grown crops but, these resources are often not available to growers (Koike, 2015). Field diagnosis of Fusarium wilt is complicated by the fact that other soilborne diseases exhibit very similar symptoms for example, *Phytophthora cactorum* is able to cause severe crown and leather rot

in strawberry (Erwin, 1996). Therefore, a method is required to accurately distinguish the pathogen and verify if it is a formae speciales of *F. oxysporum*. To do this, the genetic material (genome) of the subject pathogen would require testing.

In this research project we aim to identify the full range of genetic variants in *F. oxysporum fragariae* using novel genotyping, next generation sequencing and computing approaches. We will be using *F. oxysporum fragariae* and *lactucae* to develop a model for characterising mutations between races. Using DNA extraction techniques, we aim to extract high molecular weight DNA which will be sequenced, assembled and annotated with computing programs to develop a high-quality reference genome. Prior to this research, sequence data for *F. oxysporum fragariae* and *lactucae* was generated. However, it was not from high molecular DNA therefore, repeat-rich regions in the genome like LS regions may not have been accurately assembled from the sequence data. In addition, the improvement of existing and development novel of genome analysis programs has increased the quality of the genomes generated through these analysis pipelines.

Ultimately, this will allow the use genotyping technologies to develop a process to identify formae speciales in a quick and cost-effective test.

Project aims:

- Identify full range of genetic variants in *Fusarium oxysporum* f. sp.
- Identify epigenetic modifications which vary between the *F. oxysporum* f. sp. and races
- Use a model system to develop pipelines for identifying mutations

Materials and methods

Known methods for culturing fungal mycelia were utilised to grow both *F. oxysporum fragariae* and *lactucae* in a lab environment. Protocol for DNA extraction were derived from the protocols to extract DNA from plant materials.

Culture: Fungal material

Samples of F. oxysporum f. sp. were received from collaborators in the USA, UK (Warwick) and China. Each sample was inoculated on a nutrient-rich (potato dextrose) agar plate and placed in a dark incubator for up to 4 days. A plug of the ‘leading edge’ was excised using a

2mm cork-borer and grown in 50 mL nutrient (PDB) broth shaking at 185rpm for up to 4 days in the dark.

For the growth curve experiments, three identical flasks were set up for each timepoint, from the same mycelial plate. 10 µl of the mycelial growth was taken and viewed under a light microscope to observe growth.

Mycelia were isolated from the nutrient broth by using a vacuum pump and filter paper. The tissue was then snap-frozen in liquid nitrogen and freeze-dried in 1.5 ml Eppendorf tubes overnight to calculate dry mass after x days of growth. The dry fungal mass was then stored at -20ºC until use for DNA extraction. This was done in triplicate for the growth curve experiments to generate a mean value for fungal dry mass for each timepoint for the isolates being studied.

DNA extraction

Schalamun method:

The plant DNA extraction method by Schalamun (Schalamun, 2017) was followed with the addition of PVPP when grinding the tissue under liquid nitrogen to remove contaminants. A premade lysis buffer (500mM NaCl, 1% PvP-40,100 mM Tris-HCl pH 8, 500mM EDTA, 1.25% Sodium dodecyl sulfate (SDS), 1% Sodium Metbabisulfite, 5 mM dithiothreitol (DTT) and dH_2O) was heated to 64°C for 30 minutes then cooled to room temperature (RT). 1 µL RNase A was added per 1 mL lysis buffer. Dry fungal tissue was weighed and PVPP was added at 10% and ground in a pre-cooled mortar with a pestle. The ground tissue was placed in a 15 ml falcon tube and lysis buffer was added. The mixture was inverted until there were no visible frozen lumps remaining and was incubated at 37°C in a thermomixer shaking at 600 rpm for 20 min. Proteinase K was added, and the tubes were placed on the thermomixer again for 20 min. Followed by ice for 5 minutes. 0.3 volumes of 5M Potassium Acetate was added, mixed by inversion then immediately kept on ice at ~4°C. The samples were centrifuged at 8000g for 12 min at 4°C and the supernatant was transferred to a new tube while attempting not to disturb the pellet. 1 volume of AMPure XP beads was added to the supernatant. It was then mixed by inversion and left to incubate on a rotor for 10 minutes at RT. The tubes were spun down for 1 second and placed on a magnetic rack for 10 minutes to draw the DNA bound to the beads towards the magnet. The supernatant was discarded without disturbing the beads and1 mL of fresh 70% Ethanol was used to wash the beads. This ethanol was removed, and the step was repeated. The tubes were spun for 1 sec and placed on the magnetic rack to remove the remaining ethanol. The beads were air dried for 30 sec. 100 µL of low TE buffer (10 mM Tris-HCl pH 8 + 0.1 mM EDTA pH 8) at 50°C was added to the beads and the tube

was flicked to resuspend the beads in the buffer. The tubes were rotated for 10 minutes at RT. Following this, they were spun down for 1 sec and placed on the magnet rack for 10 min. The supernatant was transferred to a fresh Eppendorf tube and analyses were run to check the quality and the concentration of the DNA. The samples from the same isolate were usually pooled at this point.

Further clean-up was conducted if the quality of DNA was insufficient. Low TE would be added to the sample to make 500 µL. 500 µL Chloroform: Isoamylalcohol (24:1) was then added and the tube was inverted about 100 times (~ 2 min). Tubes were spun to separate the phases at 8000g at 4°C for 10 min, the upper aqueous phase (DNA) was transferred to a new tube and the chloroform phase was discarded. This step was repeat 2-4 times. 50 µL (0.1 V) 3 M Sodium Acetate (NaAc) was added and mixed by inverting the tube. 500 µL (1 V) of ice-cold 100% ethanol added and mixed by inverting tube carefully a few times and then let incubate on ice for 5 - 10 minutes. DNA was then placed in a centrifuge at 4°C and 5000g for 2 min to precipitate HMW DNA. The supernatant was carefully moved to a new tube and put on ice. The pellet was washed in 1 mL 70% ethanol in a centrifuge at 4°C and 8000g for 5 minutes. This step would be repeated 2-3 times with fresh ethanol each spin. After the ethanol wash, the supernatant was discarded, and the pellet was left to airdry for 5 min. The pellet was dissolved in 50 µL of low TE buffer at 50°C and further quality check analyses were conducted. Additional chloroform extractions were carried out if necessary.

Schwessinger method:

This extraction is based on the protocol of Schwessinger (Schwessinger, 2019). Premade buffers were combined to form lysis buffer (Buffer A; 0.35 M sorbitol, 0.1 M TrisHCl, 5 mM EDTA pH 8 Buffer B; 0.2 M Tris-HCl, 50 mM EDTA pH 8, 2 M NaCl, 2% CTAB Buffer C; 5% filter-sterile Sarkosyl N-lauroylsarcosine sodium salt) which was briefly heated to 64°C and 10 µL (10kU) RNAse T1 was added to lysis buffer once it had cooled to RT. Fungal tissue was ground in liquid nitrogen as above. The ground tissue was placed in a 50 ml falcon tube and 17.5 mL of lysis buffer was added. Mixture was left to incubate at RT for 30 min gently inverting at 5-min intervals. 40 µL of Proteinase K was added and the incubation step was repeated. After this, DNA was chilled on ice for 5 minutes before 3.5 mL 5 M potassium acetate was added and placed back on ice for 5 min. Samples were then centrifuged for at 5000 g and 4°C for 12 min. The supernatant was removed to a fresh falcon tube. 1 volume (17.5 mL) of Phenol: Chloroform: Isoamylalcohol (P/C/I) (25:24:1) was added to the supernatant and inverted for 2 min. The samples were then spun in a centrifuge at 4000 g and 4°C for 10 min. The P/C/I step was repeated 2-3 times until the upper aqueous phase was clear. The upper aqueous phase (~17 mL) was transferred to a fresh falcon tube and 5 µL of RNase T1 was added, the mixture was left to incubate for 30 min. 0.1 volume of 3.5 M

Sodium acetate was added and mixed by inverting. Followed by 1 volume of isopropanol, the mixture was incubated for 10 min at RT. The sample was then spun at 7200 g and 4°C for 45 minutes. The supernatant was transferred and preserved, and the pellet was transferred to an Eppendorf tube using a sterile 1 mL pipette cut tip with the remaining liquid. The pellet was then spun at 13,000 g and 4°C for 5 min, the supernatant was discarded, and 1 mL of 70% ethanol was added. The tube was inverted to dislodge the supernatant from the base, it was then spun again at 13,000 g and 4°C for 5 min. The alcohol washing and centrifugation step was repeated twice at most. The ethanol and excess solution were removed. The pellet was left to air dry for 7 min, after that time, 200 µL of 10 mM Tris-HCl pH 9 heated to 50°C was added and left to dissolve for 3 hours. A further 200 µL of low TE heated to 50°C was added and the tube was flicked to mix the two buffers. The mixture was left at RT overnight. 100 µL of low TE was added and the mixture was incubated at 1400 RPM and 28°C for 1 hour on the thermo mixer. Quality check analyses were conducted and if the DNA didn't meet the quality threshold required, further chloroform:IAA extractions were carried out as described above for the Schalamun method.

Nanopore sequencing

For sequencing on the GridION © (Oxford Nanopore Technology) the Nanopore Protocol "Genomic DNA by ligation (SQK-LSK109)" was followed with minor adjustments. The sample was incubated the cycler for 30 min at 37°C with flicking every 2 min as opposed to 5 minutes at 20°C during the final library elution stage. The flow cell was primed with the Flow Cell Priming Kit (EXP-FLP002). Loading beads were not used when priming the SpotON flow cell. Excluding these changes, the protocol was followed precisely.

Results

DNA extractions were first conducted using the Schalamun method to observe the quality of DNA produced. The 'purity' of the DNA produced was not sufficient to use in further steps. The Schwessinger method was then implemented as it was formulated to extract HMW DNA from fungi. This method produced DNA with higher quality DNA shown by the ratios of absorbance compared to the Schalamun method as shown in the figures below. However, this was still not ideal for sequencing as the ratios inferred that there were contaminants present in the extraction product. As a result of this, growth curve experiments were conducted to identify the log phase in the growth of fungi. Using this data, it identified the optimum time to harvest DNA from fungal tissue.

Different quality checking analyses are used to quantify the size and concentration of DNA in preparation for sequencing. To determine the size and integrity of the DNA, the Agilent 4200 TapeStation system was used. Thermo NanoDrop ND-1000 UV/VIS Spectrophotometer machine was used to measure the concentration of DNA and the quality of DNA. It does this by measuring ratios of absorbance at 260/280 and 260/230nm wavelengths of light. This in turn indicates the quality of DNA. Values between 2-2.2 for 260/230 ratios and 1.8-2.0 for 260/280 indicate pure DNA with no contaminants. DNA concentrations were also measured and compared with Thermo Invitrogen Qubit 2.0 Fluorometer. Another indication of good quality DNA with no contaminants should have concentration at similar values for both Nanodrop and Qubit measurements.

DNA extractions

Schalamun method

The method by Schalamun was the first DNA extraction protocol attempted. *F. oxysporum lactucae* race 1 & 4 were first used to ascertain the efficiency of this protocol for DNA extractions for fungi. The fungal material was grown for a week. The first set of extraction experiments gave the values shown in Table 1 on a NanoDrop™ Spectrophotometer reading. The 260/280 ratios in Table 1 show “pure” DNA with ratios near 1.8 however, the 260/230 ratios indicate the presence of contaminants. This assessment was conducted on the 27/02/20. I had yet to learn how to use the Qubit or Tapestation at this point therefore, there are no recordings of size or DNA concentration.

Table 1. Schalamun DNA extraction results for *F. oxysporum (Fo) lactucae.*

Stage	Schalamun DNA extractions					
	Crude extraction			Post Chloroform: IAA clean up		
Sample	Concentration (ng/µL)	260/280	260/230	Concentration (ng/µL)	260/280	260/230
Fo lac race 1	42.9	1.80	0.80	5.4	1.88	0.62
Fo lac race 4	109.2	1.76	1.16	19.4	1.71	0.98

Due to the concentration and ratio values produced in Table 1, the protocol was edited by splitting the sample after adding Protienase K and an increase in the time of centrifugation steps. This was rerun with the aim of optimising the extraction for fungal DNA. In this instance, only *F. oxysporum lactucae* race 4 was tested on 04/03/20. There are no differences between

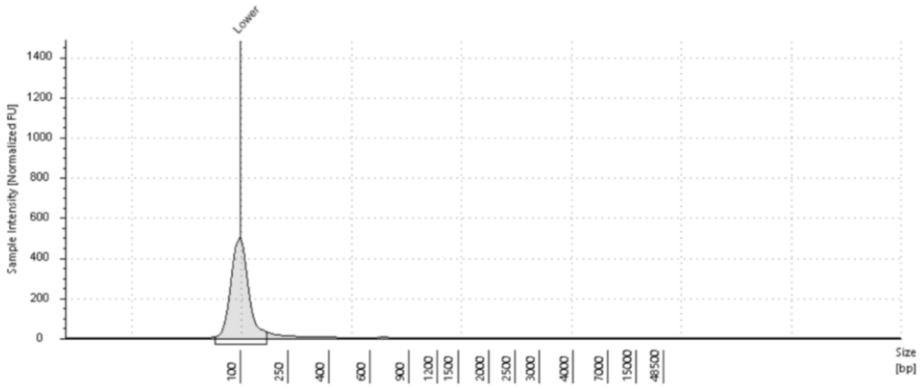
samples in Table 2 they were labelled this way to differentiate clearly between the non-pooled samples.

Table 2. DNA extractions for *F. oxysporum lactucae* (*Fol*) race 4

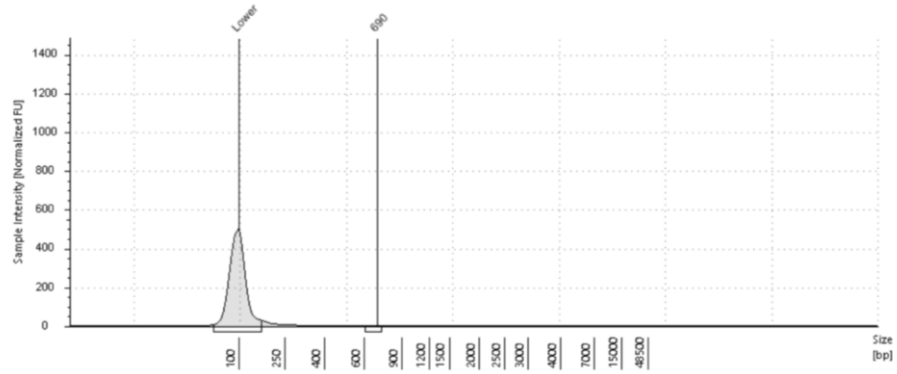
Schalamun DNA extractions						
Stage	Post Chloroform: IAA clean up					
Sample	Concentration (ng/µL)	260/280	260/230	Qubit (ng/µL)	Size via TS analysis (bases)	DNA integrity number
Fol R4 A1	1.2	2.93	0.04	>0.5	166	-
Fol R4 A2	2.2	1.23	0.06	>0.5	725	-
Fol R4 B1	208	1.88	1.51	3.44	14657- >60000	7.2
Fol R4 B2	140.4	1.8	1.84	5.86	>60000	8.5

From the data shown in Table 1 & 2, there is a noticeable decrease in the DNA purity ratios of the *Fol* R4 A samples. The Screen tape analysis from the Tapestation showed samples B1 & B2 had DNA greater than 60 kilobases (kb) in size after the further clean up. DNA integrity number (DIN) is generated by an algorithm to determine the fragmentation of the DNA, the closer the score is to 10 it suggests minimal fragmentation and shearing of the DNA. The low concentrations samples A1 and A2 did not allow a DIN score to be generated. To observe any differences that might occur between differences between formae speciales, this protocol was also conducted on 6 *F. oxysporum fragariae* isolates.

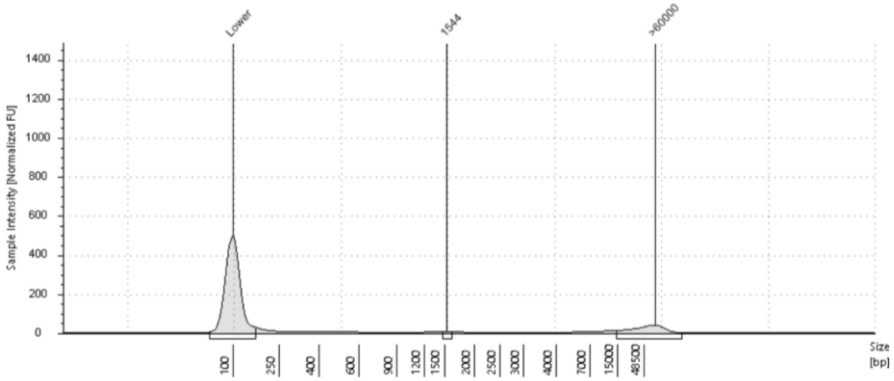
1a. **B1: A1 AJ516 post PCI clean**



1b. **C1: A2 AJ516 post PCI clean**



1c. **D1: B1 AJ516 post PCI clean**



1d. **E1: B2 AJ516 post PCI clean**

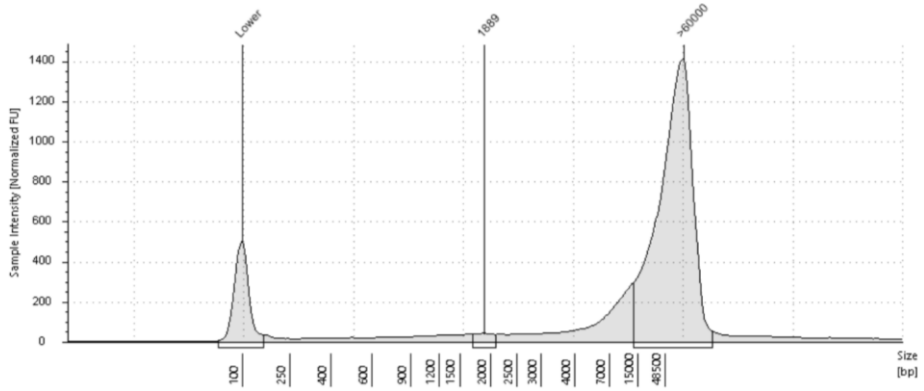


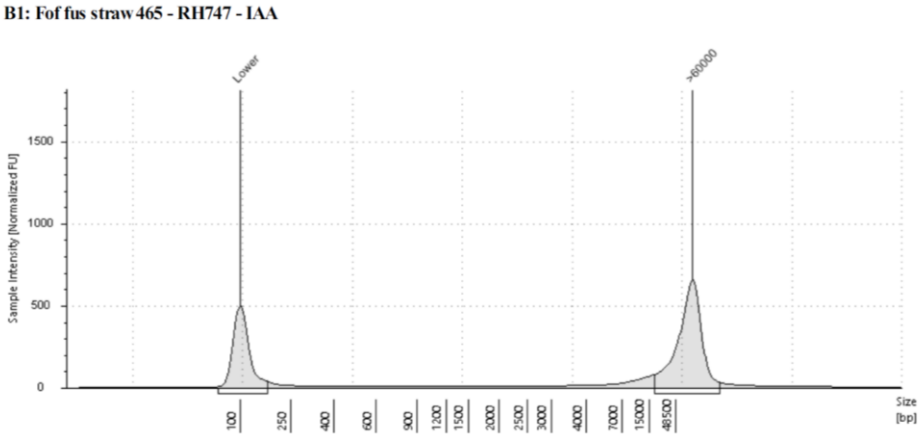
Figure 1a-1d display the molecular weight plots from the extraction products in Table 2. Figure 1a shows sample A1 no DNA above ~200 bases and no HMW DNA. A2 had a very similar graph generated however, showed DNA reaching the length of 690 bases which is also small. 1c shows a slight peak at DNA >60 kb the area under the peak indicates a low concentration of DNA this size. Sample B2 depicted in figure 1d shows a much higher peak at >60 kb indicating the presence of HMW DNA. This method was conducted on the *F. oxysporum fragariae* isolates.

Table 3. DNA extractions for *F. oxysporum fragariae (Fof)* isolates using the Schalamun method

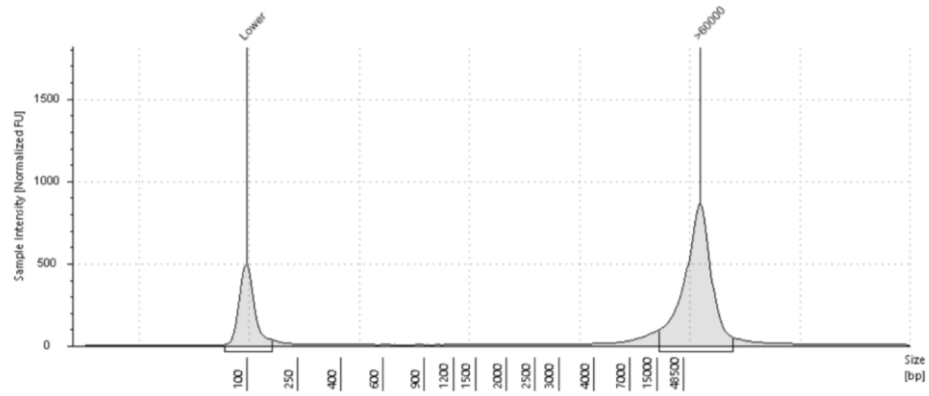
Schalamun DNA extractions						
Stage	Post Chloroform: IAA clean up					
Fof isolate	Concentration (ng/µL)	260/280	260/230	Qubit (ng/µL)	Size via TS analysis (bases)	DIN
Fus straw 465	24.7	1.88	1.7	30	17921->60000	8.9
15.004	65.4	1.7	0.97	39.6	15913->60000	9.5
15.074	41.7	1.72	1.03	28.6	14262 ->60000	9.4
14.003	14.3	1.66	0.99	9.38	15621->60000	9.0
15.041	27.9	1.84	1.01	15.9	16525->60000	9.5
PC41/17	16.5	1.72	0.94	65.8	18899->60000	9.0

The data in Table 3 from the Fus straw 465 shows DNA of sufficient size range through the TS analysis results, quality and integrity for sequencing after cleaning. However, the other samples appear to be contaminated given that most values for the 260/230 ratios are around 1.00. This experiment was conducted on the 26/6/20 and the data presented shows the Schalamun method was not ideal for these extractions without further modifications to the protocol. The analysis from the Tapestation depicted all of these samples to have DNA in the region of 60 kb. This is a reasonable indication that the DNA in the samples are HMW.

2a.



2b. **C1: Fof 15.004 - RH748 -IAA**



2c. **D1: Fof 15.074 - RH749 -IAA**

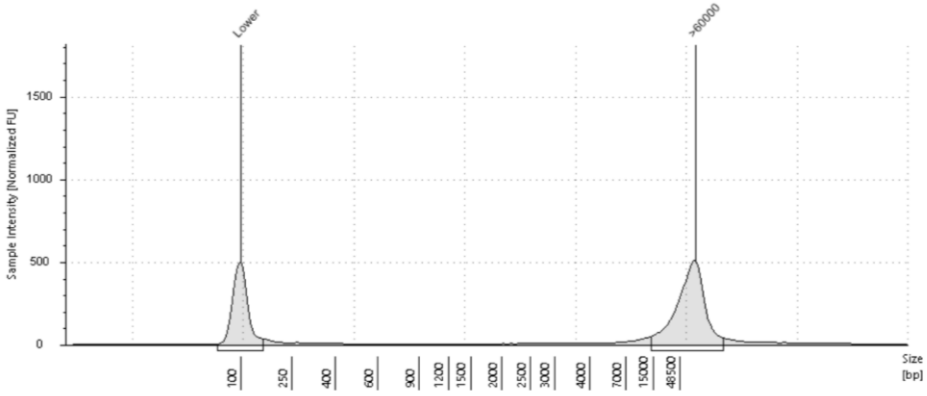


Figure 2 shows the size plots of the samples in table 3. All the products display peaks above 60kb. Samples 14.003, 15.041 and PC41/17 have curves identical to that of 2c. From the graphs generated, isolates Fus straw 465 and 15.004 have the largest concentration of HMW DNA present amongst the products of this extraction.

Schwessinger method:

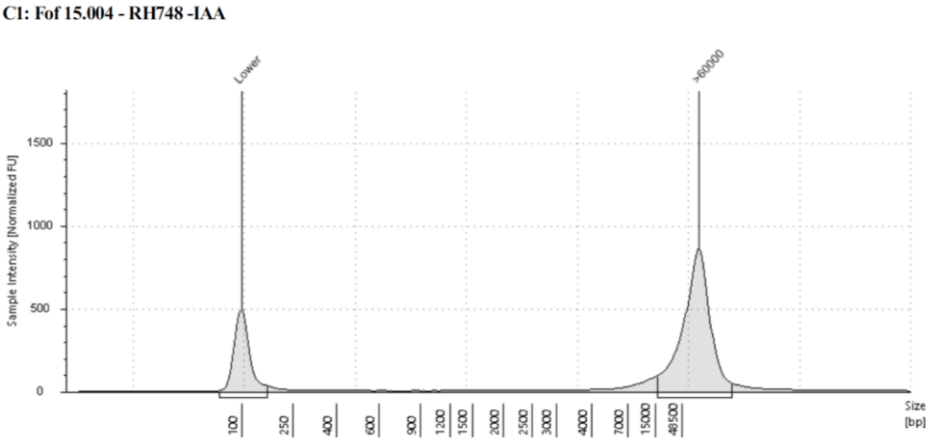
This protocol was first used on 4 *F. oxysporum fragariae* isolates on the date 12/06/20. It was then carried out on the 2 F. oxysporum *lactucae* isolates on the 23/06/20 to see if this protocol would produce high quality, HMW DNA. The results in Table 4 show a decrease in the concentration of the DNA present after cleaning. The clean-up step did improve the quality of the DNA as shown by the 260/280 and 260/230 ratios, for most samples there was a visible increase in the value generated for the ratios. This trend excludes the sample 14.003 which shows a decrease.

Table 4. DNA extractions for *F. oxysporum* (*Fo*) isolates using the Schwessinger method

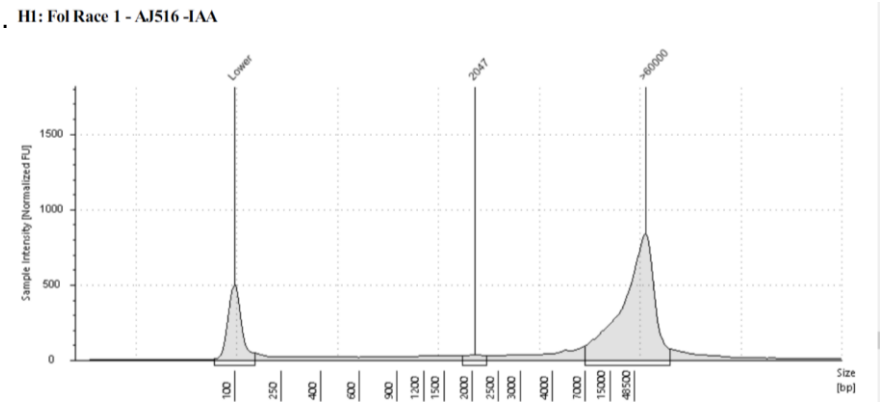
Schwessinger DNA extractions						
Stage	Post Chloroform: IAA clean up					
Fo sample	Concentration (ng/µL)	260/280	260/230	Qubit (ng/µL)	Size via TS analysis (bases)	DIN
Fof 15.004	34.4	1.87	1.71	27.6	7268->60000	8.3
Fof 15.074	22.3	1.86	1.83	14.5	13863->60000	7.0
Fof 14.003	18.9	1.63	0.98	7.1	17153->60000	7.2
Fof 15.041	18.9	1.85	1.89	14.7	10471->60000	7.6
Fol Race 1	63.3	1.87	1.94	72	7014->60000	7.8
Fol Race 4	91	1.83	1.58	86.8	18553->60000	9.2

The graphs produced by the Tapestation analyses for *F. oxysporum fragariae* isolates were very similar to Figure 3a. The graphs generated all indicate DNA above 60kb however, the DIN scores below 9.0 suggest fragmentation and shearing of the DNA which is not ideal for sequencing. Figure 3b and 3c depict the resultant graphs for *F. oxysporum lactucae* race 1 & 4.

3a.



3b.



3c. A2: Fol Race 4 - AJ520-1AA

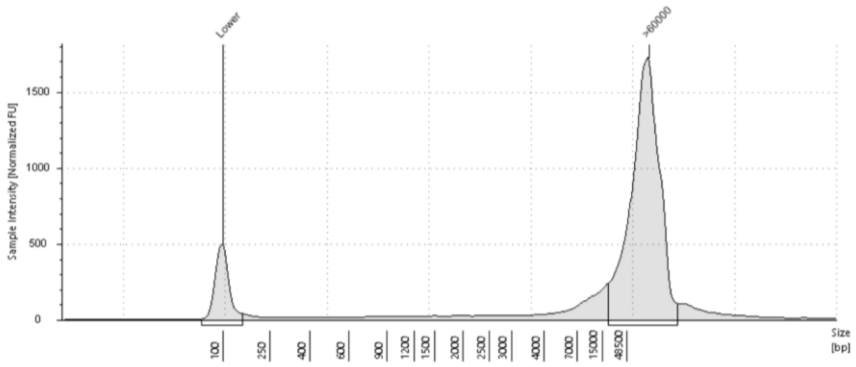
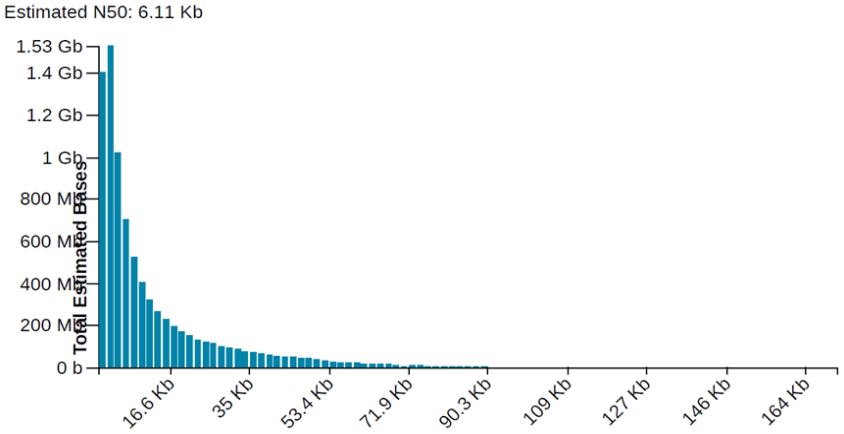


Figure 3b and 3c show a trail after the peak of >60kb indicating HMW DNA ~80kb but there is also a trail connecting the "lower" peak and the ">60kb" peak also showing a wide range of DNA sizes present in the sample.

Nanopore sequencing

Extracted DNA from *F. oxysporum lactucae* race 1 was sequenced on the GridION ©, DNA for 16 hours generating 2.86 million sequence reads and generated 7.85 Gb data. The results in Figure 4 show half of the genome sequence is covered by contigs larger than or equal 6.11kb. This value is known as the N50. The mean read length was 2.7kb which is very small. 6.11kb and 2.7kb are very small values and are not considered HMW DNA. The longest read length however was 164kb which is HMW DNA which was promising for a first attempt. Due to the length of time taken, sequencing was done overnight from 03/07/20 to the next morning. Histogram in Figure 4 shows there are many shorter DNA reads than there are HMW DNA reads. After observing the sequence data summary, the supposed HMW DNA gave mostly short reads. Thus, growth curve experiments were conducted to improve sampling of the extracted DNA.

Figure 4. Histogram of estimated base length for *F. oxysporum lactucae* race 1



Growth curve

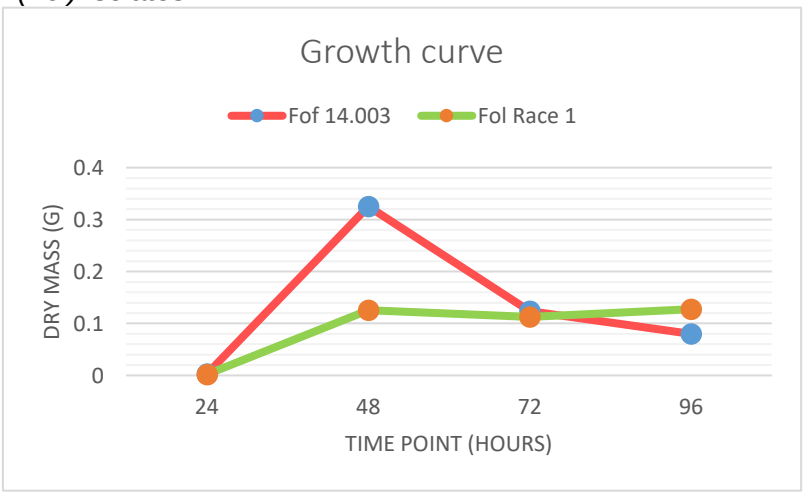
The first attempts at DNA extraction were carried out on fungal cultures after 7 days of growth. The fungal culture had stopped growing by this point and was at stationary phase. It was thought that DNA quality may be improved by taking samples at an earlier point in the growth curve. To further optimise the DNA extraction process, a growth curve was generated from the cultured mycelium to identify the log phase in fungal growth for this formae speciales. *F. oxysporum lactucae* race 1 and *F. oxysporum fragariae* isolate 14.003 were chosen as representatives for each f sp. Table 5 shows a steep increase in the dry fungal mass between 24 and 48 hours, this was designated as time in which exponential growth occurs.

Table 5. Contrasts between *F. oxysporum fragariae (Fof) and lactucae (Fol)* isolates in dry fungal mass

	Dry mass of fungi (grams)											
	24 hrs			48 hrs			72 hrs			96 hrs		
Sample	1	2	3	1	2	3	1	2	3	1	2	3
Fof 14.003	0.003	0.003	0.002	0.304	0.279	0.393	0.039	0.154	0.179	0.101	0.059	X
Fol Race 1	0.002	0.001	0.003	0.13	0.133	0.113	0.124	0.101	0.112	0.114	0.033	0.141

The grey “x” in sample 3 represents a sample that was lost in the process of incubation. For *F. oxysporum fragariae* 14.003 there is a decline in dry fungal mass after 48 hours which was plotted in Figure 5. *F. oxysporum lactucae* Race 1 has an exponential increase between 24 and 48 hours however, plateaus afterwards with a slight increase at the final timepoint. The decrease in fungal mass for the *F. oxysporum fragariae* sample was an unexpected result considering the steep increase between the first two time points. This experiment took place between 31/08 - 4/09/20.

Figure 5. Graph depicting the dry fungal mass of *F. oxysporum fragariae (Fof) and lactucae (Fol)* isolates



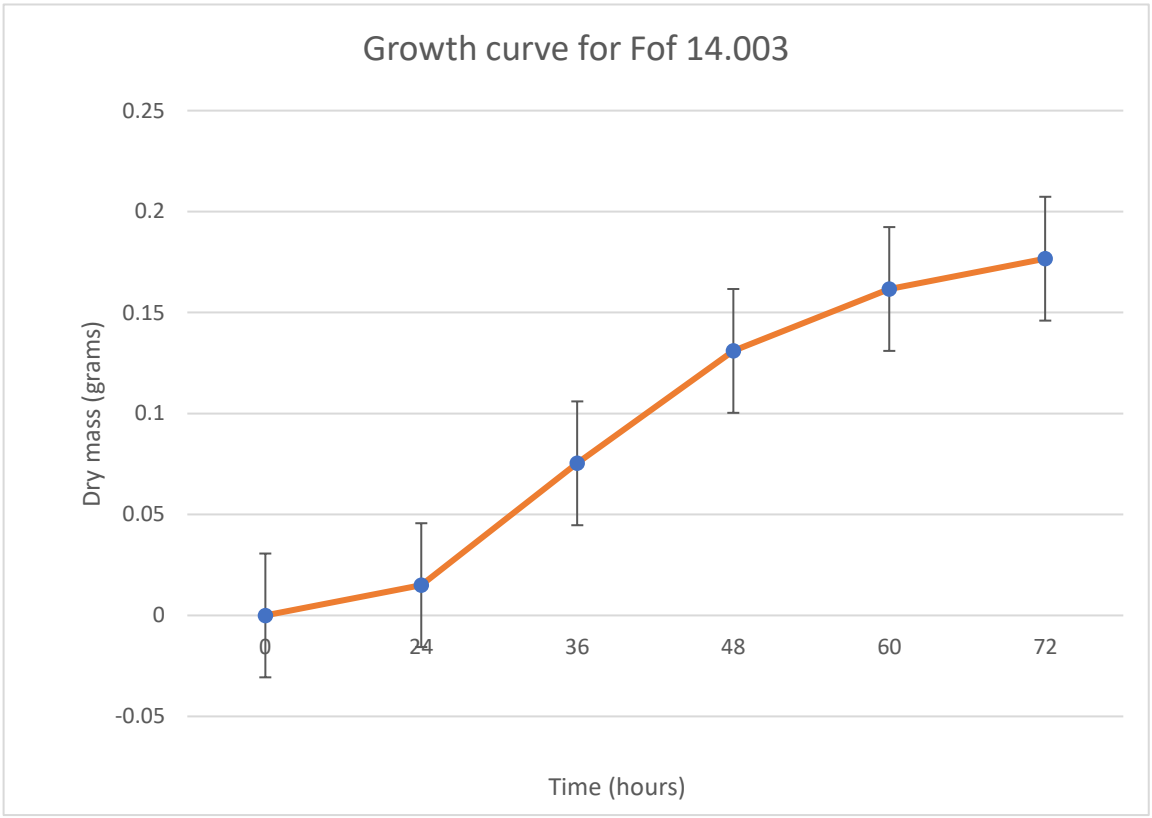
Following this experiment, another was designed with shorter increments between time points using only *F. oxysporum fragariae* 14.003 shown in Table 6 and Figure 6.

Table 6. Dry fungal mass of *F. oxysporum f sp. fragariae (Fof)* isolate 14.003

Time point	Dry fungal mass (grams)														
	24 hrs			36 hrs			48 hrs			60 hrs			72 hrs		
Repeat	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Fof 14.003	0.015	0.015	0.015	0.088	0.065	0.073	0.14	0.123	0.13	0.168	0.138	0.179	0.181	0.165	0.184

The experiment was repeated to identify the peak growth during the exponential phase and to observe if a decrease in dry fungal matter re-occurred. The curve in Figure 5 shows a steep increase in mass between 24-36 hours and 36-48 hours. The rate of increase begins to plateau from 60-72 hours. Figure 6 shows no reduction in dry fungal mass which is contrary to data shown in Figure 5.

Figure 6. Graph depicting the dry fungal mass of *F. oxysporum fragariae* isolate 14.003 over time



The observation from these results has led to the conclusion that for future DNA extractions, DNA should be harvested from fungal tissue no longer than 48 hours in this media. The curve may vary depending on the medium used for growth.

Bioinformatics

Prior to sequencing, previous sequence data (before this project) was analysed and run through a data analysis pipeline. The programs run have been used to assemble, polish and quality check the genome. Following this, different analyses to identify candidate genes, particularly effectors, secreted proteins and transmembrane proteins. The different programs have also been used to locate potential orthologs. Orthologs are genes that exist in different species yet have the same function as they have descended from a common ancestor. This pipeline will be updated and applied to the sequence data produced later in this project.

Discussion

This report presents preliminary data of a 4-year PhD project. Two different DNA extraction techniques, Schalamun and Schwessinger protocol, were compared and optimised. The aim is to extract high molecular weight DNA which will be sequenced, assembled and annotated with computing programs to develop a high-quality reference genome. DNA extraction protocols designed specifically to isolate DNA from an organism while simultaneously removing contaminants present.

As the Schalamun protocol was intended for DNA extraction from Eucalyptus plant tissue which has toxic chemicals which damage DNA, it was applied and tested on fungal tissue. Sample *F. oxysporum lactucae* R4 B1 in Table 2 and *F. oxysporum* isolate Fus straw 465 in Table 3 had good results utilising this extraction protocol nonetheless, they were only 2 out of the 8 samples. 260/230 ratios are a secondary indication of DNA purity by measuring the contamination of DNA. Ideally, for sequencing samples should have a 260/280 value $x>1.8$ and a 260/230 between 2-2.20. Of the remaining samples, ratio values for 260/230 were around 1.00 which is an insufficient ratio value to consider for sequencing. The results from the Schalamun method did not meet the desired quality for sequencing which was not unexpected. Plant and fungal material differ in their composition, meaning that the Schalamun protocol was designed to remove contaminants that may arise from the resultant plant material during the process. Therefore, in this case this method may not be completely effective for producing fungal genomic DNA as fungal material may not be removed contaminating the DNA product.

The Schwessinger protocol was developed for extracting DNA from fungal matter. This explains in why the values presented in Table 4 exceed the concentration and purity ratio values shown in Table 2 & 3. As the reagents in each lysis buffer differs, the effect it has on the fungal tissue and DNA will also vary. HMW DNA is fragile, so sheering DNA by vortexing and pipetting is quite common. In addition, large amounts of extracted DNA were being lost in the ethanol precipitation step.

In the first year of this project, the optimum time of sampling was investigated. Replicate growth curve experiments were conducted to identify log phase of fungal growth (optimal growth period) prior to harvesting mycelia. The data infers the exponential phase of fungal growth is between 24-48 hours. Utilising this information, culture time prior to DNA extraction will be reduced from 96 hours to 36 or 48 hours. In this phase mycelial plugs are growing and replicating, with fewer senescent or dying cells, and less build-up of toxic secondary metabolites, leading to isolation of high quality HMW DNA.

DNA extractions are a crucial step in this sequence of experiments. To reduce the loss of DNA, specific steps were taken. It was observed that rotating DNA with Sodium acetate for 30 min to 1 hour rather than 10 min (as stated in the protocol) gave DNA more time to react with reagents prior to the ethanol precipitation step. This centrifugation step was also extended to ensure pelleting of HMW DNA. Supernatants potentially retaining HMW DNA were kept and the quality and size was checked to see if the DNA could be pooled with the sample. HMW DNA is fragile, therefore wide-bore pipette tips were used to reduce turbulence caused by the suction force in turn reducing potential shearing of DNA.

Sequencing of the whole genomes and transcriptomes will be conducted so that comparisons can be made between *F. oxysporum fragariae* isolates and *F. oxysporum lactucae races* to identify mutations which affect their pathogenicity. The current sequence data of *F. oxysporum lactucae* requires further analysis to annotate and analyse the features identified from computational analysis. The sequence data is being processed through informatics programs which simplify the management and handling of the large amounts of sequence data.

Conclusions

Fusarium wilt can be a recurring issue causing significant financial losses for growers in trying to manage it in soil, which can become a problem during the propagation chain. This project aims to design a model for accurate identification of Fusarium wilt pathogens which could

later be applied to other crop pathogens. Early identification will allow effective management strategies to be implemented earlier to increase crop yield.

The experiments conducted to date are to assist further research:

- Growth curves for *F. oxysporum f sp. fragariae* and *lactucae* have determined optimum time for sampling in DNA extractions
- DNA extraction methods are being optimised
- Informatics analysis programs are being developed to analyse sequence data

Future work will include:

- Pathogenicity tests racially ambiguous *F. oxysporum fragariae* isolates
- Nanopore sequencing of remaining samples
- RNA sequence experiments to develop a transcriptome

Knowledge and Technology Transfer

AHDB PhD conference – Nottingham – January 2020: Delivered presentation

NIAB EMR Lab meeting – Kent – June 2020: Delivered Project update

Oxford Nanopore's London Calling Conference – Online – June 2020: Conference attendee

References

Agriculture, C. D. (2019, December). *California Agricultural Production Statistics.* Retrieved from CDFA: https://www.cdfa.ca.gov/statistics/

Erwin, D. C. (1996). *Phytophthora Diseases Worldwide.* St. Paul, Minnesota, USA: American Phytopathological Society.

Geiser, D. M. (2013, May). One fungus, one name: defining the genus Fusarium in a scientifically robust way that preserves longstanding use. *Phytopathology, 103*(5), 400-408. doi:10.1094/PHYTO-07-12-0150-LE

Gordon, T. R. (2017, May 10). Fusarium oxysporum and the Fusarium Wilt Syndrome. *Phytopathology, 55*, 23-39. doi:doi.org/10.1146/annurev-phyto-080615-095919

Henry, P. M. (2020, September). The potential forFusarium oxysporum f. sp. fragariae, cause of Fusarium wilt of strawberry, to colonize organic matter in soil and persist through anaerobic soil disinfestation. *Plant Pathology, 69*(7), 1218-1226. doi:10.1111/ppa.13225

Koike, S. T. (2015, July). Management of Fusarium wilt of strawberry. *Crop protection, 73*, 67-72. doi:10.1016/j.cropro.2015.02.003

Mayjonade, B. G. (2016, October). High molecular weight gDNA extraction. *Bio techniques, 61*(4), 203-205.

Puri, S. S. (2019). Mycotoxins: A Threat to Food Security and Health. *International Journal of Applied Sciences and Biotechnology, 7*(3), 298–303. doi:10.3126/ijasbt.v7i3.24651

Schalamun, M. &. (2017, October 15). *High molecular weight gDNA extraction after Mayjonade et al. optimised for eucalyptus for nanopore sequencing.* doi:dx.doi.org/10.17504/protocols.io.ka2csge

Schwessinger, B. (2019, May 17). *High quality DNA from Fungi for long read sequencing e.g. PacBio.* doi:10.17504/protocols.io.2yfgftn

Taylor, A. A. (2019, December 19). Basal Rot of Narcissus: Understanding Pathogenicity in Fusarium oxysporum f. sp. narcissi. *Frontiers in Microbiology, 10*, 2905. doi:10.3389/fmicb.2019.02905

Taylor, A. J. (2019). *First Report of Fusarium oxysporum f. sp. lactucae Race 4 Causing Lettuce Wilt in England and Ireland.* St. Paul, Minnesota: American Phytopathological Society Publications. doi:10.1094/PDIS-10-18-1858-PDN

Upasani, M. L. (2016, May 26). Dynamics of Colonization and Expression of Pathogenicity Related Genes in Fusarium oxysporum f.sp. ciceri during Chickpea Vascular Wilt Disease Progression. *PLoS One, 11*(5), e0156490. doi:10.1371/journal.pone.0156490

Winks, B. L. (1965). A wilt of strawberry caused by a new form of Fusarium oxysporum. *Queensland Journal of Agricultural and Animal Sciences, 22*, 475-479.

